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On the influence of the rhodamine substituents onto the cytotoxicity of mitocanic maslinic acid rhodamine conjugates

Marie Kozubek, Toni C. Denner, Marc Eckert, Sophie Hoenke, René Csuk *

Organic Chemistry, Martin-Luther University Halle-Wittenberg, Kurt-Mothes-Str. 2, D-06120 Halle (Saale), Germany

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ABSTRACT

Maslinic acid was converted via a di-acetylated piperazinyl amide into rhodamine conjugates differing in their alkyl moieties. These conjugates were submitted to cytotoxicity assays employing a panel of human tumor cell lines. These conjugates held high cytotoxicity but also some selectivity especially for A2780 cells. Thereby, a propyl substituted rhodamine conjugate showed EC_{50} values as low as $EC_{50} = 0.01$ μM and was approx. 15 times more cytotoxic for the cancer cells than for non-malignant fibroblasts (NIH 3 T3). Cytotoxicity obviously parallels the lipophilicity of the residue and suggests - since the compounds act as mitocanes - an interaction of the conjugates with the inner mitochondrial membrane.

Introduction

Compounds that selectively address mitochondria of cancer cells are currently considered an innovative and promising option for cancer chemotherapy. [1–12] This is partly due to the discovery that mitochondria are more than just the "power plants" of cells. [13–18].

Some time ago, we showed that pentacyclic triterpenes are ideal starting materials for the development of cytotoxic compounds. [19–22] Thereby, we revealed that the following prerequisites must be present: an amide-bound spacer between the carboxyl group of the triterpene and a distal cationic group. While "simple" quaternary ammonium salts showed improved cytotoxicity as compared to the parent compounds, a breakthrough could be achieved by accessing lipophilic rhodamine B derivatives. The use of a piperazinyl spacer proved to be particularly advantageous. [23–32].

While a hybrid of acetylated oleanolic acid (Fig. 1) with piperazinyl spacer and rhodamine B already showed good cytotoxicity towards human tumor cells, the corresponding analogue from maslinic acid, [33] which - in comparison to oleanolic acid - also carries an additional hydroxyl group at C-2, was clearly more cytotoxic; at the same time the latter compound showed a higher selectivity towards tumor cells in comparison to non-malignant cells (NIH 3 T3). [21] This trend of better cytotoxicity was also observed for the corresponding benzylamides. The benzylamide of 3-*O*-acetyloleanolic acid showed an EC_{50} of 4.3 μM for A2780 human ovarian adenocarcinoma cells, while the benzylamide of di-acetylated maslinic acid (**EM2**) held a significantly higher cytotoxicity ($EC_{50} = 0.5$ μM) for the same cell line. [34] The same applies to the acetylated piperazinyl amides. After already having carried out investigations on the spacer (ethylenediamine or piperazine), the piperazinyl spacer proved to be beneficial for obtained low EC_{50} values. Thus, an investigation of the influence of the distal rhodamine residue [25] was called for.

Results and discussion

Several routes have been suggested for the synthesis of substituted rhodamines. [35,36] Due to good commercial availability of the starting material and the shortness of the route (Scheme 1), we decided to use 3-aminophenol as a starting material, whose reaction with alkyl-halides gave the dialkyl-3-aminophenols **4**–**7**. The rhodamines **8**–**11** were accessed from the reaction of **4**–**7** with phthalic anhydride in the presence of catalytic amounts of aluminum trichloride.

Maslinic acid (**1**) was extracted from pitted olives as previously described; [34,37] its acetylation (Scheme 2) gave known di-acetate **2**. [33] The reaction of **2** with oxalyl chloride in the presence of catalytic amounts of dimethylformamide (DMF) followed by a reaction with piperazine furnished piperazinyl-amide **3**. [30].

The reaction of **8**–**11** with oxalyl chloride converted the rhodamines *in situ* into the corresponding acid chlorides; these were allowed to react with **3** to afford the piperazinyl-spacered triterpene-rhodamine conjugates **12**–**15**.

To assess the cytotoxicity of these compounds sulforhodamine B

* Corresponding author.
E-mail address: rene.csuk@chemie.uni-halle.de (R. Csuk).

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(SRB) assays were performed employing several human tumor cell lines. The results from these assays as well the tumor/non-tumor cell selectivity S [$EC_{50\ (NIH\ 3T3)}$ / $EC_{50\ (respective\ cell\ line)}$] are summarized in Table 1.

As a result, compound **1** was cytotoxic to only a minor extent; much stronger cytotoxicity was observed for piperazinyl amide **3**. This compound is cytotoxic to all tumor cell lines but also to non-malignant fibroblasts (NIH 3 T3) to about the same extent. In contrast, a marked increase in cytotoxicity up to an approximately 100-fold factor was observed for the rhodamine conjugates **12**–**14**. All compounds show a particular cytotoxicity for A2780 cells (EC_{50} 0.02 to 0.01 μM). However, **14** is the most cytotoxic for both A2780, A375 and MCF-7 cells, while a much weaker cytotoxicity was observed for NIH 3 T3 cells. This is also reflected in the calculated selectivity S (S = $EC_{50,\ NIH\ 3T3}$ / $EC_{50\ respective\ cell\ line}$). The cell selectivity is highest (S = 15.0) for A2780 cells. In principle, the cytotoxicity seems to increase with a longer chain length of the alkyl substituent on the rhodamine moiety. This also correlates well with the calculated log $p_{octanol/water}$ partition coefficients for the rhodamine-piperazinyl residues: this coefficient increases from 0.61 (for methyl-substitution) to 1.72 (for ethyl) to 3.05 (for propyl). Thus, there seems to be a certain - but not conclusively clarified - correlation between the substitution pattern on the rhodamine and the observed cytotoxicity. Earlier we could show that triterpene-piperazinyl-rhodamine conjugates are to be considered and act as mitocanes and their cytotoxic effect is probably due to an interaction with the inner mitochondrial membrane.

Conclusion

Maslinic acid (from the extraction of pitted olives) was acetylated and converted into the corresponding piperazinyl amide **3** whose coupling with rhodamines differing in their alkyl moieties led to the formation of triterpene-rhodamine conjugates **12**–**15**. These conjugates were cytotoxic to a panel of human tumor cell lines but less to non-malignant fibroblasts. Worthwhile to mention that these compounds held some selectivity for A2780 cells, and especially compound **14**, a propyl substituted rhodamine conjugate showed EC_{50} values as low as EC_{50} = 0.01 μM and was approx. 15 times more cytotoxic for the cancer cells than for the fibroblasts. The measured cytotoxicity obviously parallels the calculated octanol/water partition coefficient and suggests - since the compounds act as mitocanes - an interaction with the inner mitochondrial membrane.

Experimental

General

NMR spectra were recorded using the Varian spectrometers DD2 and VNMRS (400 and 500 MHz, respectively). MS spectra were taken on a Advion expression^{L} CMS mass spectrometer (positive or negative ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2 μA, capillary temperature: 250 °C, capillary voltage: 180 V, sheath gas: N2). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel. IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin-Elmer. The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin-Elmer. The optical rotations were measured either on a JASCO P-2000 or a Perkin-Elmer polarimeter 341 at 20 °C. The melting points were determined using the Leica hot stage microscope Galen III and are uncorrected. Elemental analyses were performed on a Foss-Heraeus Vario EL (CHNS) unit. The solvents were dried according to usual procedures.

Biological testing

Cell lines and culture conditions

Following human cancer cell lines A375 (malignant melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast cancer), A2780 (ovarian carcinoma), HeLa (cervical cancer) and NIH 3 T3 (non-malignant mouse fibroblasts) were used. All cell lines were obtained from the Department of Oncology (Martin-Luther-University Halle Wittenberg). Cultures were maintained as monolayers in RPMI 1640 medium with L-glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10 % heat-inactivated fetal bovine serum (Sigma-Aldrich GmbH, Steinheim, Germany) and penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37 °C in a humidified atmosphere with 5 % CO_2.

Cytotoxicity assay (SRB assay)

For the evaluation of the cytotoxicity of the compounds the sulforhodamine-B (Kiton-Red S, ABCR GmbH, Karlsruhe, Germany) micro-culture colorimetric assay was used. The EC_{50} values were averaged from three independent experiments performed each in triplicate and calculated from semi-logarithmic dose–response curves applying a non-linear 4P Hills-slope equation.

Syntheses

General procedure a (GPA)

3-Aminophenol (7.6 g, 69.6 mmol) was dissolved in DMF (50 mL) and the respective alkyl halide (205 mmol) and potassium carbonate (18.0 g, 130 mmol) were added; stirring was continued at 100 °C for 3–8 h. Usual aqueous workup followed by chromatographic purification furnished compounds **4**–**7**.

General procedure B (GPB)

The rhodamines **8**–**11** were synthesized by heating a mixture of the respective dialkyl-3-aminophenol **4**–**7** (28.4–41.4 mmol), phthalic

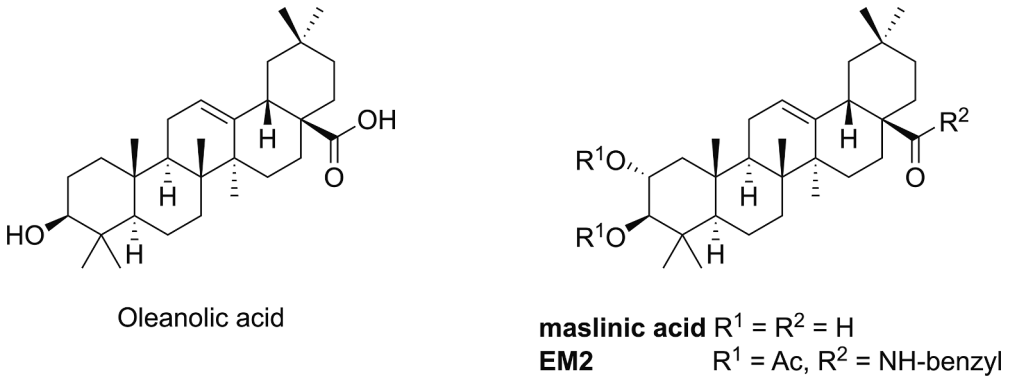


Fig. 1. Structure of oleanolic acid (OA), maslinic acid (MA, 1) and cytotoxic derivative EM2.

anhydride (14.2 – 20.7 mmol) and a catalytic amount of aluminum trichloride to 200 °C for 5–60 min (completion of the reaction checked by TLC). After the completion of the reaction the crude product was purified by column chromatography.

General procedure C (GPC)

Compounds **8**–**11** (0.4 mmol) were dissolved each in dry DCM (10 mL) and at 0 °C oxalyl chloride (0.1 mL, 1.4 mmol) and a catalytic amount of DMF were added, stirring continued at room temperature for 2 h. The volatiles were removed under reduced pressure, and the residue was re-dissolved with THF (3 × 10 mL), and the solvent was removed again. The residue was dissolved in dry DCM (5 mL) and added to a solution of compound **3** (330 mg, 0.5 mmol), triethylamine (0.9 mL, 0.7 mmol) and a catalytic amount of DMAP in dry DCM (5 mL); the mixture was stirred at room temperature for 24 h. The solvent was removed, and the residue subjected to chromatography to yield **12**–**15**.

*Maslinic acid (**1**)*

Pitted green olives (bought from a local discounter, 10 kg) were crushed into small pieces and dried for 2 days at 130–135 °C. The dry material (2.6 kg) was suspended in methanol (3 L) and allowed to stand (with occasional swaying) for 2 days. The mixture was filtered, and the filter cake was extracted with methanol (each 3 L, 2 days, procedure repeated 3 times). The solvent was removed, and the residue subjected to chromatography (silica gel, *n*-hexane/ethyl acetate/methanol, 5:5:1). re-crystallization (*n*-hexane/ethyl acetate) yielded **1** (13.8 g) as a colorless solid; m.p. 264–267 °C (decomp.), (lit.: [38] 265–268 °C (decomp.); R_F = 0.36 (*n*-hexane/ethyl acetate, 1:2).

*2α, 3β-Bis(acetyloxy)-olean-12-en-28-oic acid (**2**)*

Maslinic acid (1) was acetylated as previously described followed by a chromatographic purification of the crude product (silica gel, *n*-hexane/ethyl acetate, 9:1) to yield **2** (78 %); m.p. 280–283 °C (lit.: [33] 287–289 °C); R_F = 0.31 (silica gel, *n*-hexane/ethyl acetate, 6:4).

*2α, 3β-Bis(acetyloxy)-olean-12-en-28-oyl piperazine (**3**)*

Compound **2** was converted into its piperazinyl amide **3** as previously reported in 86 % yield. m.p. 156–159 °C (lit.: [30] 157–160 °C); R_F = 0.35 (silica gel, chloroform/methanol, 9:1).

*3-(Dimethylamino)phenol (**4**)*

According to GPA from methyl iodide followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 4:1) **4** (52 %) was obtained as a white solid; m.p. 82–85 °C (lit: [39] 84–85 °C); R_F = 0.55 (chloroform/methanol, 95:5).

*3-(Diethylamino)phenol (**5**)*

According to GPA from ethyl bromide followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 9:1), **5** (67 %) was obtained as an off-white solid; m.p. 52–55 °C (lit.: [40] 55 °C); R_F = 0.42 (chloroform/methanol, 95:5).

*3-(Dipropylamino)phenol (**6**)*

According to GPA from *n*-propyl bromide followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 9:1) **6** (61 %) was obtained as an off-white solid; m.p. 98–99 °C (lit: [41] 99.7–100.1 °C); R_F = 0.37 (chloroform/methanol, 95:5).

*3-(Dibenzylamino)phenol (**7**)*

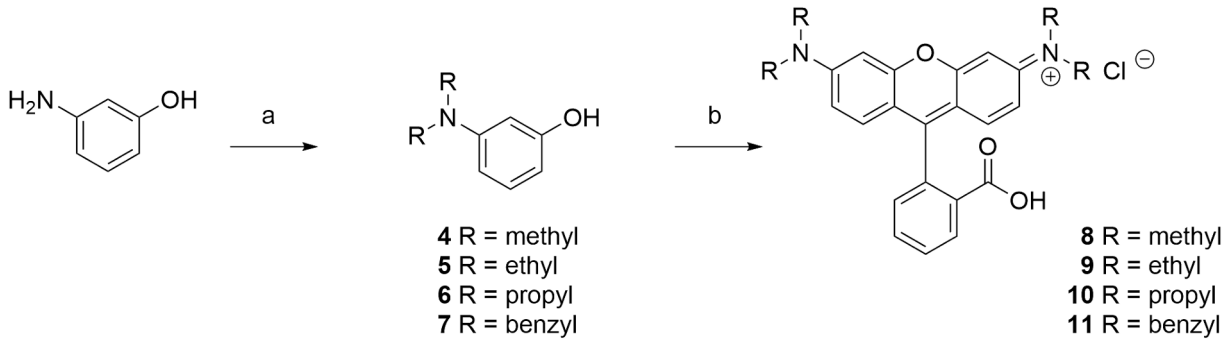
According to GPA with benzyl bromide (25 mL) followed by chromatography (silica gel, *n*-hexane/ethyl acetate, 9:1) **7** (8.3 g, 41 %) was obtained as a white solid; m.p. 61–63 °C (lit.: [42] 62–64 °C); R_F = 0.68 (chloroform/methanol, 95:5); IR (ATR): ν = 3514br, 3387br, 3084w, 3061 m, 3027 m, 2905 m, 2865 m, 1703 m, 1615 s, 1603 s, 1578 s, 1502 s, 1494 s, 1451 s, 1395 s, 1359 s, 1328 s, 1297 s, 1277 s, 1262 s, 1166 s, 1074 s, 1044 m, 1027 s, 1001w cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.42 – 7.18 (m, 10*H*, 9-H + 10-H + 11-H), 7.01 (t, *J* = 8.1 Hz, 1H, 5-H), 6.37 (d, *J* = 5.4 Hz, 1H, 6-H), 6.26 (br s, 1H, 2H), 4.63 (s, 4H, 7-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 156.8 (C-1), 151.0 (C-3), 138.5 (C-8), 130.3 (C-5), 128.8 (C-10), 127.1 (C-11), 126.8 (C-9), 105.5 (C-4), 104.0 (C-6), 99.7 (C-2), 54.3 (C-7) ppm; MS (ESI, MeOH/chloroform, 4:1): *m/z* = 288.1 (56 %, $[M-H]^-$), 290.1 (40 %, $[M + H]^+$).

*9-(2-Carboxyphenyl)-3,6-bis(dimethylamino)xanthylium chloride (**8**)*

According to GPB from **4** followed by chromatography (silica gel, chloroform/MeOH, 9:1) **8** (35 %) was obtained as a violet solid; [43] m. p. 250 °C; R_F = 0.12 (chloroform/methanol, 9:1); UV–vis (MeOH): λ^{max} (log ε) = 254 (3.81), 354 (3.30), 541 (4.28) nm; IR (ATR): ν = 3362br, 2925br, 1718 m, 1645 s, 1590 s, 1537 s, 1514 s, 1490 s, 1407 s, 1364 s, 1346 s, 1262 m, 1220 s, 1187 s, 1138 s, 1090 m, 1071 s cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): δ = 7.96 (d, *J* = 7.6 Hz, 1H, 3-H), 7.77 (td, *J* = 7.5, 1.0 Hz, 1H, 5-H), 7.70 (td, *J* = 7.6 Hz, 1H, 4-H), 7.20 (d, *J* = 7.6 Hz, 1H, 6-H), 6.64 – 6.33 (m, 6H, 10-H + 10′-H + 11-H + 11′-H + 13-H + 13′-H), 2.93 (s, 12H, 15-H + 15‘-H) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 168.9 (C-1), 152.5 (C-7), 152.2 (C-14), 152.2 (C-14′), 151.9 (C-12), 151.9 (C-12′), 135.3 (C-5), 129.9 (C-4), 128.3 (C-10), 128.3 (C-10′), 126.6 (C-2), 124.4 (C-3), 124.0 (C-6), 109.0 (C-11), 109.0 (C-11′), 106.1 (C-9), 106.1 (C-9′), 98.0 (C-13), 98.0 (C-13‘), 84.7 (C-8), 39.8 (C-15), 39.8 (C-15‘) ppm; MS (ESI, methanol/chloroform 4:1): *m/z* = 387.2 (64 %, $[M-Cl]^+$), 409.2 (24 %, $[M-Cl + Na]^+$).

*9-(2-Carboxyphenyl)-3,6-bis(diethylamino)xanthylium chloride (**9**)*

According to GPBA from **5** followed by chromatography (silica gel, chloroform/MeOH, 12:1) **8** (45 %) was obtained as a violet solid; m.p. 163–166 °C; R_F = 0.25 (chloroform/methanol, 9:1); identical with commercial material (m.p., m.m.p., ^1H and ^{13}C NMR).



Scheme 1. Reactions and conditions of the rhodamine synthesis: (a) DMF, potassium carbonate and methyl iodide (→4 (52 %)), ethyl bromide (→5 (67 %)), *n*-propyl bromide (→6 (61 %)), or benzyl bromide (→7 (41 %)), 3–8 h, 21 °C; (b) phthalic anhydride, aluminum trichloride (cat.), 5–60 min, 200 °C, yield: 8 (35 %), 9 (45 %), 10 (42 %), 11 (35 %).

*9-(2-Carboxyphenyl)-3,6-bis(dipropylamino)xanthylium chloride (**10**)*

According to GPB from **6** followed by chromatography (silica gel, chloroform/MeOH, 12:1) **10** (42 %) was obtained as a violet solid; m.p. 185–188 °C; R_F = 0.34 (chloroform/methanol, 9:1); UV–vis (MeOH): λ^{max} (log ε) = 224 (4.47), 259 (4.48), 284 (4.18), 305 (4.17), 550 (4.95) nm; IR (ATR): ν = 2957 m, 2931 m, 2872 m, 1741 s, 1637 s, 1614 s, 1589 s, 1544 s, 1519 s, 1464 s, 1430 s, 1410 s, 1370 s, 1337 s, 1286 s, 1265 s, 1232 s, 1216 s, 1196 s, 1180 s, 1157 m, 1126 s, 1113 s, 1102 s, 1038w, 1010 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.21 (d, *J* = 8.4 Hz, 1H, 3-H), 7.65 – 7.55 (m, 2H, 5-H + 4-H), 7.19 – 7.13 (m, 1H, 6-H), 6.91 (d, *J* = 9.0 Hz, 2H, 10-H + 10'-H), 6.61 – 6.52 (m, 4H, 13-H + 13'-H + 11-H + 11'-H), 3.39 – 3.33 (m, 8H, 15-H + 15'-H), 1.67 (dt, *J* = 15.3, 7.6 Hz, 8H, 16-H + 16'-H), 0.95 (t, *J* = 7.4 Hz, 12H, 17-H + 17'-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 168.3 (C-1), 156.1 (C-7), 155.6 (C-14), 155.6 (C-14'), 152.8 (C-12), 152.8 (C-12'), 132.9 (C-5), 130.8 (C-7), 130.4 (C-4), 130.4 (C-10), 130.4 (C-10′), 129.6 (C-3), 128.9 (C-2), 126.6 (C-6), 113.7 (C-9), 113.7 (C-9′), 111.0 (C-11), 111.0 (C-11′), 97.0 (C-13), 97.0 (C-13′), 86.6 (C-8), 53.3 (C-15), 53.3 (C-15'), 20.6 (C-16), 20.6 (C-16'), 11.4 (C-17), 11.4 (C-17') ppm; MS (ESI, methanol/ chloroform 4:1): *m/z* = 517.3 (100 %, $[M-Cl]^+$).

*9-(2-Carboxyphenyl)-3,6-bis(dibenzylamino)xanthylium chloride (**11**)*

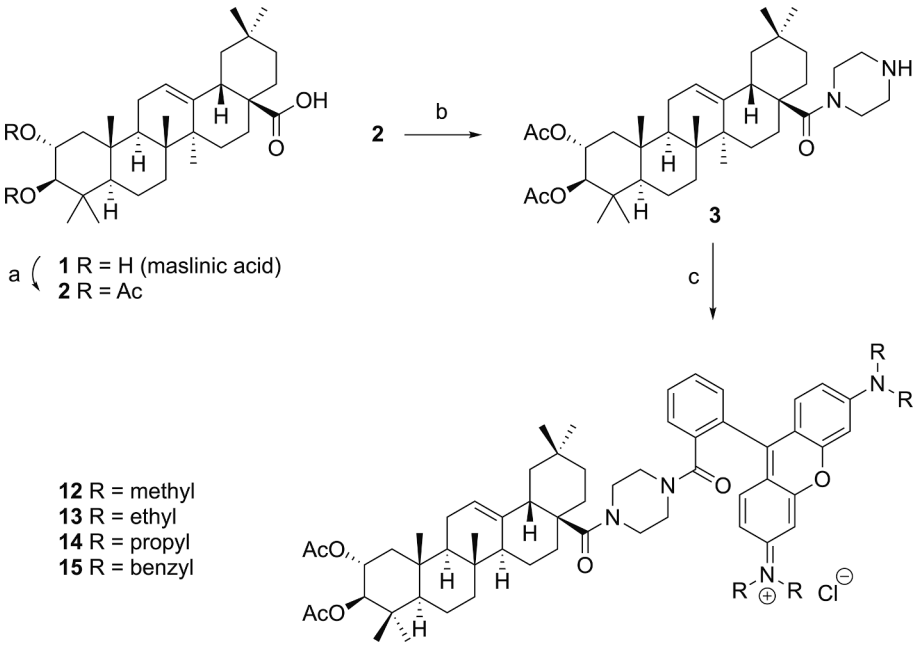
According to GPB from **7** followed by chromatography (silica gel, chloroform/MeOH, 9.8:0.2) **11** (35 %) was obtained as a violet solid; m.p. 111–114 °C; R_F = 0.60 (chloroform/methanol, 9:1); UV–vis (MeOH): λ^{max} (log ε) = 256 (4.49), 352 (4.21), 540 (4.80) nm; IR (ATR): ν = 3061w, 3028w, 2908w, 2862w, 1753 s, 1721 s, 1631 s, 1613 s, 1592 s, 1582 s, 1549 s, 1516 s, 1494 s, 1465 s, 1451 s, 1426 s, 1404 s, 1392 s, 1342 s, 1330 s, 1284 s, 1241 s, 1229 s, 1201 s, 1156 s, 1129 s, 1108 s, 1077 s, 1028 m, 1002w cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.09 (d, *J* = 9.6 Hz, 1H, 3-H), 7.62 (td, *J* = 7.5, 1.2 Hz, 1H, 5-H), 7.51 – 7.47 (td, *J* = 7.8, 1.1 Hz, 1H, 4-H), 7.35 – 7.16 (m, 20H, 18-H + 18′-H + 19-H + 19′-H + 17-H + 17′-H), 6.87 (d, *J* = 9.1 Hz, 1H, H-6), 6.75 (d, *J* = 9.9 Hz, 2H, 10-H + 10′-H), 6.64 – 6.56 (m, 4H, 13-H + 13′-H + 11-H + 11′-H), 4.71 (s, 8H, 15-H + 15′-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 165.5 (C-1), 155.8 (C-14), 155.8 (C-14'), 155.1 (C-12), 155.1 (C-12') 153.6 (C-7), 136.6 (C-16), 136.6 (C-16'), 134.9 (C-6), 133.5 (C-5), 130.3 (C-10), 130.3 (C10'), 129.8 (C-4), 129.1 (C-18), 129.1 (C-18'), 128.1 (C-2), 127.6 (C-19), 127.6 (C-19'), 127.5 (C-3), 126.5 (C-17), 126.5 (C-17'), 111.3 (C-9), 111.3 (C-9'), 111.1 (C-11), 111.1 (C-11'), 98.4 (C-13), 98.4 (C-13'), 84.9 (C-8), 54.5 (C-15), 54.5 (C-15') ppm; MS (ESI, MeOH/ chloroform 4:1) *m/z* = 713.2 (3 %, $[M-Cl + Na-2H]^-$), 691.3 (50 %, $[M-Cl]^+$); analysis calcd for $C_{48}H_{39}ClN_2O_3$ (727.30): C 79.27, H 5.41, N 3.85; found: C 78.90, H 5.63, N 3.69.

Table 1
SRB assay EC_{50} values [μM] after 72 h of treatment; averaged from three independent experiments performed each in triplicate; confidence interval CI = 95 %. Human cancer cell lines: A375 (melanoma), HT29 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), HeLa (cervical carcinoma), NIH 3 T3 (non-malignant fibroblasts); cut-off 30 μM, n.d. not determined; doxorubicin (DX) has been used as a positive standard; compound 15 was not soluble under the conditions of the assay.

[μM]	A375	HT29	MCF-7	A2780	HeLa	NIH 3 T3
MA	>30	28.8 ± 0.5	>30	19.5 ± 0.8	>30	21.1 ± 0.2
3	2.0 ± 0.1	1.6 ± 0.1	1.0 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	3.2 ± 0.02
12	0.07 ± 0.01	0.11 ± 0.04	0.05 ± 0.02	0.02 ± 0.001	0.15 ± 0.02	0.30 ± 0.04
13	0.05 ± 0.01	0.09 ± 0.03	0.03 ± 0.01	0.02 ± 0.005	0.08 ± 0.03	0.25 ± 0.03
14	0.02 ± 0.004	0.07 ± 0.02	0.03 ± 0.005	0.01 ± 0.001	0.05 ± 0.01	0.15 ± 0.04
DX	n.d.	0.9 ± 0.01	1.1 ± 0.3	0.01 ± 0.01	n.d.	0.4 ± 0.0
Selectivity						
12	4.3	2.7	6.0	15.0	2.0	
13	5.0	2.8	8.3	12.5	3.1	
14	7.5	2.1	5.0	15.0	3	

*9-[2-[[4-(2α,3β-Bis(acetyloxy)-olean-12-en-28-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(dimethylamino]-xanthylium chloride (**12**)*

According to GPC with **8** (0.2 g) followed by chromatography (silica gel, chloroform/MeOH, 9:1) **12** (168 mg, 46 %) was obtained as a violet solid; m.p. 211–214 °C; R_F = 0.32 (chloroform/methanol, 9:1); UV–vis (MeOH): λ^{max} (log ε) = 257 (4.38), 304 (4.05), 555 (4.84) nm; IR (ATR):



Scheme 2. Reactions and conditions: (a) Ac_2O, NEt_3, DMF (cat.), DCM, 21 °C, 1 day, →2 (78 %); (b) oxalyl chloride, NEt_3, DMF (cat.), DCM, 21 °C, 5 h, then piperazine, DCM, NEt_3, DMAP, 0 °C → 21 °C, 30 min, →3 (86 %); (c) rhodamines 8–11, oxalyl chloride, DMF (cat.), DCM, 0 °C → 21 °C, 2 h, then 3, NEt_3, DMAP (cat.), DCM, 21 °C, 24 h, yield: 12 (46 %), 13 (61 %), 14 (58 %), 15 (52 %).

ν = 2924 s, 2856 m, 1738 s, 1632 s, 1592 s, 1534 m, 1494 s, 1456 s, 1408 s, 1364 s, 1343 s, 1281 s, 1252 s, 1232 s, 1184 s, 1124 s, 1064 m, 1042 s, 1032 s, 1002 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.71 – 7.62 (m, 1H, 41-H), 7.55 – 7.50 (m, 1H, 39-H), 7.39 – 7.36 (m, 1H, 40-H), 7.31 – 7.27 (m, 2H, 46-H + 46′-H), 6.99 (d, J = 9.3 Hz, 2H, 47-H + 47′-H), 6.85 (d, J = 4.7 Hz, 2H, 49-H + 49′-H), 5.19 (m, 1H, 12-H), 5.06 (td, J = 11.1, 4.5 Hz, 1H, 2-H), 4.72 (d, J = 10.3 Hz, 1H, 3-H), 3.33 (s, 12H, 51-H + 51′-H), 3.27 (br s, 8H, 36-H + 35-H), 2.97 (d, J = 9.6 Hz, 1H, 18-H), 2.11 – 2.05 (m, 1H, 16-Ha), 2.03 (s, 3H, 33-H), 2.01 – 1.96 (m, 1H, 1-Ha), 1.95 (s, 3H, 32-H), 1.93 – 1.77 (m, 2H, 11-Ha + 11-Hb), 1.67 – 1.47 (m, 5H, 19-Ha + 16-Hb + 7-Ha + 6-Ha + 15-Ha), 1.46 – 1.39 (m, 1H, 22-Ha), 1.37 – 1.27 (m, 3H, 6-Hb + 21-Ha + 16-Hb), 1.27 – 1.21 (m, 2H, 7-Hb + 22-Hb), 1.18 – 1.09 (m, 2H, 21-Hb + 19-Hb), 1.08 (s, 3H, 27-H), 1.06 – 1.02 (m, 2H, 15-Hb + 1-Hb), 1.00 (s, 3H, 26-H), 0.94 (d, J = 10.3 Hz, 1H, 5-H), 0.88 (s, 6H, 23-H + 24-H), 0.86 (s, 3H, 29-H), 0.86 (s, 3H, 30-H), 0.63 (s, 3H, 25-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.7 (C-28), 170.9 (C-31), 170.6 (C-24), 167.8 (C-37), 157.6 (C-50), 157.6 (C-50‘), 157.6 (C-44), 157.5 (C-48),157.5 (C-48‘), 144.8 (C-13), 135.2 (C-43), 132.2 (C-46), 132.2 (C-46‘), 130.5 (C-42), 130.5 (C-38), 130.4 (C-40), 130.3 (C-41), 127.8 (C-39), 121.2 (C-12), 114.5 (C-47), 114.5 (C-47′), 114.1 (C-45), 114.0 (C-45′), 97.0 (C-49‘), 97.0 (C-49), 80.7 (C-3), 70.1 (C-2), 55.0 (C-5), 47.7 (C-9), 47.6 (C-36), 46.4 (C-17), 46.3 (C-19), 43.9 (C-1), 43.6 (C-18), 42.0 (C-35), 41.4 (C-51′), 41.3 (C-51), 39.4 (C-4), 39.4 (C-14), 39.2 (C-8), 38.3 (C-10), 34.0 (C-21), 33.1 (C-30), 32.7 (C-22), 30.4 (C-20), 29.8 (C-7), 28.5 (C-24), 27.9 (C-15), 25.9 (C-27), 24.1 (C-29), 23.5 (C-11), 22.8 (C-16), 21.2 (C-32), 21.0 (C-33), 18.3 (C-6), 17.7 (C-23), 17.0 (C-25), 16.5 (C-26) ppm; MS (ESI, methanol/chloroform, 4:1): m/z = 993.8 (100 %, $[M-Cl]^+$), 1027.8 (10 %, $[M-Cl + MeOH + H]^+$); analysis calcd for $C_{61}H_{79}ClN_4O_7$ (1015.77): C 72.13, H 7.84, N 5.52; found: C 71.87, H 8.03, N 5.31.

*9-[2-[[4-(2α,3β-Bis(acetyloxy)-olean-12-en-28-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(dimethylamino]-xanthylium chloride **(13)***

As previously reported from **9** in 70 % yield as a violet solid: m.p. 245–248 °C (lit.: [30] 247–249 °C); R_F = 0.30 (silica gel, chloroform/methanol, 9:1).

*9-[2-[[4-(2α,3β-Bis(acetyloxy)-olean-12-en-28-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(dipropylamino]-xanthylium chloride **(14)***

According to GPC with **10** (0.2 g) followed by chromatography (silica gel, chloroform/MeOH, 11:1) **14** (291 mg, 58 %) was obtained as a violet solid; m.p. 236–239 °C; R_F = 0.38 (chloroform/methanol, 9:1); UV–vis (methanol): λ^{max} (log ε) = 260 (4.48), 307 (4.14), 566 (5.02) nm; IR (ATR): ν = 2938 m, 2874 m, 1737 s, 1632 s, 1586 s, 1528 m, 1508 s, 1468 s, 1429 s, 1411 s, 1393 s, 1363 s, 1336 s, 1300 s, 1252 s, 1230 s, 1177 s, 1132 s, 1100 s, 1033 s, 1001 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.70 – 7.61 (m, 1H, 41-H), 7.53 – 7.50 (m, 1H, 39-H), 7.34 – 7.17 (m, 4H, 40-H + 46-H + 46‘-H + 42-H), 6.97 (d, J = 8.4 Hz, 2H, 47-H + 47‘-H), 6.74 – 6.72 (m, 2H, 49-H + 49‘-H), 5.18 (t, J = 3.3 Hz, 1H, 12-H), 5.05 (td, J = 11.0, 4.6 Hz, 1H, 2-H), 4.71 (d, J = 10.3 Hz, 1H, 3-H), 3.50 (m, 8H, 51-H + 51′-H), 3.30 (m, 8H, 36-H + 35-H), 2.97 (d, J = 13.2 Hz, 1H, 18-H), 2.11 – 2.04 (m, 1H, 16-Ha), 2.02 (s, 3H, 33-H), 1.99 – 1.94 (m, 1H, 1-Ha), 1.94 (s, 3H, 32-H), 1.91 – 1.47 (m, 16H, 11-Ha + 11-Hb + 52-H + 52′-H + 16-Hb + 19-Hb + 7-Ha + 7-Hb + 6-Ha + 15-Ha), 1.46 – 1.37 (m, 1H, 22-Ha), 1.37 – 1.26 (m, 2H, 21-Ha + 6-Hb), 1.21 (d, J = 12.7 Hz, 1H, 22-Hb), 1.15 – 1.01 (m, 4H, 21-Hb + 19-Hb + 1-Hb + 15-Hb), 1.07 (s, 3H, 27-H), 0.99 (t, J = 7.3 Hz, 15H, 53-H + 53′-H + 26-H, 0.94 (d, J = 10.6 Hz, 1H, 5-H), 0.87 (s, 6H, 23-H + 24-H), 0.85 (s, 3H, 29-H), 0.85 (s, 3H, 30-H), 0.63 (s, 3H, 25-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.8 (C-28), 170.9 (C-31), 170.6 (C-34), 167.8 (C-37), 157.8 (C-50), 157.8 (C-50‘), 156.3 (C-44), 156.1 (C-48), 156.1 (C-48‘), 144.8 (C-13), 135.2 (C-43), 132.3 (C-46), 132.3 (C-46′), 130.7 (C-38), 130.4 (C-42), 130.4 (C-40), 130.3 (C-41), 127.7 (C-39), 121.2 (C-12), 114.5 (C-47), 114.5 (C-47′), 114.0 (C-45), 114.0 (C-45′), 96.6 (C-49‘), 96.5 (C-49), 80.7 (C-3), 70.1 (C-2), 55.0 (C-5), 53.9 (C-51′), 53.8 (C-51), 47.7 (C-9), 47.6 (C-36), 47.6 (C-17), 46.3 (C-19), 43.91 (C-1), 43.6 (C-18), 42.0 (C-35), 39.4 (C-4), 39.4 (C-14), 39.2 (C-8), 38.3 (C-10), 34.0 (C-21), 33.0 (C-30), 32.7 (C-22), 30.4 (C-20), 29.9 (C-7), 28.5 (C-24), 27.8 (C-15), 25.9 (C-27), 24.1 (C-29), 23.5 (C-11), 22.5 (C-16), 21.2 (C-32), 21.0 (C-33), 20.9 (C-52), 20.9 (C-52‘), 18.3 (C-6), 17.7 (C-23), 17.0 (C-25), 16.5 (C-26), 11.4 (C-53), 11.4 (C-53‘) ppm; MS (ESI, MeOH/chloroform 4:1): m/z = 1106.0 (100 %, $[M-Cl]^+$); analysis calcd for $C_{69}H_{95}ClN_4O_7$ (1127.99): C 73.47, H 8.49, N 4.97; found: C 73.14, H 8.68, N 4.75.

*9-[2-[[4-(2α,3β-Bis(acetyloxy)-olean-12-en-28-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(dibenzylamino]-xanthylium chloride **(15)***

According to GPC with **11** (followed by chromatography (silica gel, chloroform/MeOH, 9.5:0.5) **15** (52 %) was obtained as a violet solid; m.p. 216–219 °C; R_F = 0.45 (chloroform/methanol, 9:1); UV–vis (MeOH): λ^{max} (log ε) = 259 (4.61), 302 (4.25), 556 (5.06) nm; IR (ATR): ν = 2941 m, 2863 m, 1737 s, 1633 s, 1590 s, 1580 s, 1550 m, 1525 m, 1480 s, 1451 s, 1426 s, 1409 s, 1388 s, 1341 s, 1298 s, 1281 s, 1252 s, 1252 s, 1221 s, 1181 s, 1152 s, 1079 m, 1041 s, 1029 s, 1002 s cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.67 – 7.63 (m, 1H, 41-H), 7.53 – 7.51 (m, 1H, 39-H), 7.40 – 7.38 (m, 2H, H-46 + H-46′), 7.36 – 7.17 (m, 21H, 40-H + 42-H + 54-H + 54‘-H + 52-H + 52′-H + 53-H + 53′-H), 7.12 – 7.10 (m, 2H, 47-H + 47‘-H), 6.88 – 6.83 (m, 2H, 49-H + 49‘-H), 5.23 (t, J = 3.5 Hz, 1H, 12-H), 5.07 (td, J = 11.1, 4.7 Hz, 1H, 2-H), 4.95 – 4.68 (m, 9H, 51-H + 51‘-H + 3-H), 3.51 – 3.34 (m, 3H, 35-Ha + 36-Ha + 36-Hb), 3.25 (br s, 1H, 35-Hb), 3.04 – 2.99 (m, 1H, 18-H), 2.14 – 2.04 (m, 1H, 16-Ha), 2.03 (s, 3H, 33-H), 2.02 – 1.97 (m, 1H, 1-Ha), 1.95 (s, 3H, 32-H), 1.94 – 1.77 (m, 2H, 11-Ha + 11-Hb), 1.70 – 1.46 (m, 5H, 16-Hb + 19-Ha + 7-Ha + 7-Hb + 15-Ha + 6-Ha), 1.43 (dd, J = 12.3, 2.6 Hz, 1H, 22-Ha), 1.39 – 1.27 (m, 2H, 6-Hb + 21-Ha), 1.25 (d, J = 12.7 Hz, 1H, 22-Hb), 1.18 – 1.12 (m, 2H, 19-Hb + 21-Hb), 1.10 (s, 3H, 27-H), 1.10 – 1.01 (m, 2H, 15-Hb + 1-Hb), 1.01 (s, 3H, 26-H), 0.95 (d, J = 8.0 Hz, 1H, 5-H), 0.89 (s, 3H, 29-H), 0.87 (s, 9H, 23-H + 24-H + 30-H), 0.68 (s, 3H, 25-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.6 (C-28), 170.9 (C-31), 170.6 (C-34), 167.5 (C-37), 158.0 (C-50), 158.0 (C-50‘), 157.9 (C-44), 157.7 (C-48), 157.7 (C-48‘), 144.9 (C-13), 134.9 (C-43), 134.7 (C-52), 134.6 (C-52‘), 132.7 (C-46), 132.7 (C-46‘), 131.0 (C-38), 130.7 (C-42), 130.5 (C-40), 130.4 (C-41), 129.4 (C-54), 129.4 (C-54), 128.3 (C-55′), 128.3 (C-55), 127.9 (C-39), 126.5 (C-53′), 126.5 (C-53), 121.2 (C-12), 115.3 (C-47), 115.3 (C-47′), 115.0 (C-45), 115.0 (C-45′), 97.8 (C-49′), 97.7 (C-49), 80.7 (C-3), 70.1 (C-2), 55.2 (C-51), 55.2 (C-51′), 55.0 (C-5), 47.7 (C-9), 47.6 (C-36), 47.6 (C-17), 46.4 (C-19), 43.9 (C-1), 43.7 (C-18), 42.0 (C-35), 39.4 (C-4), 39.4 (C-14), 39.2 (C-8), 38.3 (C-10), 34.0 (C-21), 33.0 (C-30), 32.7 (C-22), 30.4 (C-20), 29.8 (C-7), 28.5 (C-24), 27.9 (C-15), 25.9 (C-27), 24.1 (C-29), 23.5 (C-11), 22.6 (C-16), 21.2 (C-32), 21.0 (C-33), 18.3 (C-6), 17.7 (C-23), 17.0 (C-25), 16.6 (C-26) ppm; MS (ESI, MeOH/chloroform, 4:1): m/z = 1298.7 (32 %, $[M-Cl + H]^+$), 1297.7 (29 %, $[M-Cl]^+$); analysis calcd for $C_{85}H_{95}ClN_4O_7$ (1320.17): C 77.33, H 7.25, N 4.24; found: C 76.97, H 7.65, N 3.97.

CRediT authorship contribution statement

Marie Kozubek: Investigation. **Toni C. Denner:** Investigation. **Marc Eckert:** Investigation. **Sophie Hoenke:** Investigation. **René Csuk:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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